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CHEMICAL NATURE OF STONE CELLS FROM PEAR FRUIT

INTRODUCTION

SCLEREIDS or stone cells are contained in the pulp of most pear varieties where they impart a gritty texture. Early studies of these "tartarecious grains" revealed they were similar in paracrystallinity to fiber cells (Crist and Batjer, 1931) being composed of successive thin layers. The intractable solubility properties of these particles led Crist and Batjer to suggest they were lignocellulosic in nature. Similarly, Smith (1935) observed that stone cells were insoluble after extraction with benzene, alcohol, alkali and water and concluded they were chemically analogous to wood lignin. There is, however, no definitive information on the chemical composition of stone cells.

We have observed an inverse relationship between free phenolic substances, mainly chlorogenic acid, and the quantity and size of stone cells in several pear varieties (Ranadive and Haard, 1971). This finding led to a suggestion that the cellular localization of peroxidase delimits the metabolism of phenolic substances into lignin (Ranadive and Haard, 1972). A practicable outgrowth of these studies is that calcium nutrition and uptake of the tree may relate the development of stone cells in pears. The present investigation was undertaken to discern the chemical nature of stone cells.

MATERIALS & METHODS

Isolation of stone cells

A 200-g sample of peeled and cored pear fruit (*Pyrus Serotina,* var. "Yuzuhada") was homogenized with 500 ml distilled water in a Waring Blendor for 5 min. The homogenate was diluted with 1,000 ml water and made 0.1M with NaCl. The suspension was incubated for 30 min at 20—22°C and the supernatant phase decanted. The sediment was incubated for 30 min with 500 ml of 0.5N NaOH and decanted. Finally, the sediment was suspended in 500 ml of 0.5N HCl for 30 min, decanted and washed with water. The above washing operations were repeated several times until the stone cells were free of extraneous cell debris.

Histochemical test for stone cells

Stone cells were observed to form a bright red conjugate when incubated with 1% phloroglucinol in 3N HCl. A similar chromophore develops when wood lignin is treated with this reagent. (Johansen, 1940).

Oxidation of stone cells

Nitrobenzene oxidation of stone cells was carried out according to the methods described for analysis of wood lignin (Stone and Blundell, 1951). The stone cells were pulverized to pass through 125 micron sieve. A 40-mg sample of stone cell powder, 0.06 ml of nitrobenzene and 2 ml of 2N NaOH were heated at 160°C for 5 hr in a sealed stainless steel bomb (25 ml capacity). The cooled mixture was filtered, brought to slightly acidic pH with HCl and analyzed by chromatography.

Oxidation was also carried out using cupric sulphate instead of nitrobenzene to determine the oxidation products of nitrobenzene itself.

Paper chromatography of oxidation products of stone cells

Unidirectional descending paper chromatography was used for separation of oxidation products. A 25 to 50 $\mu\ell$ sample was spotted on Whatman No. 1 paper. Chromatograms were developed in each of the following systems: (1) butanol:acetic acid:water (4:1:5) (2) butanol:ethanol:water (5:1:4); and (3) water saturated with benzene (Fukumuzi, 1960). Papers were developed for 16—18 hr in the case of solvent systems (1) and (2) and 3.5—4 hr with the third solvent system. The papers were air dried and sprayed with chromogenic reagents. Chromogenic reagents used were: (1) 1:1 mixture of potassium ferricyanide and $FeCl_3$ (0.5%); (2) Diazosulfanilic acid; (3) 2,4-dichlorophenylhydrazine; and (4) 2,4-dichlorophenolindophenol (Fukumuzi, 1960).

Thin layer chromatography of oxidation products of stone cells

Unidirectional thin layer chromatography was carried out on 8 in. × 8 in. thin layer plates of Silica gel G. A 25 $\mu\ell$ sample was applied to plates which were developed in each of three solvent systems: (1) benzene:ethanol (150:22); (2) benzene:acetone (3:2); and (3) methanol:chloroform (3:7) (Barton, 1967).

After drying, the plates were sprayed with a 1:1 mixture of $K_3Fe(CN)_6$ and $FeCl_3$ (0.5%) or diazosulfanilic acid.

Infrared spectroscopy of stone cells

15—20 mg of stone cell powder, passed through a 120 micron sieve, was mixed and pulverized with approximately 70 mg potassium bromide, dried with a hair dryer to remove traces of water and pressed to form a pellet. Samples were examined with a Perkin Elmer model 421 grating infrared spectrophotometer.

Table 1—Paper chromatography of nitrobenzene oxidation products

Solvent[a]	Rf	Diazosulfanilic acid	Color reactions $K_3Fe(CN)_6$ $FeCl_3$	2,6-dichloro-indolphenol	Relative conc
(1) Sample	0.91	Yellow	Blue	–	+++
	0.88	Pinkish-orange	Blue	–	+++
	0.86	Orange-pink	Blue	–	+++
	0.83	Brown	Blue	–	++
	0.82	Purple	Blue	–	+
	0.78	Orange	Blue	–	+
	0.71	Brown-tan	Blue	–	+
	0.01	Brown	–	–	+
p-hydroxybenzaldehyde	0.94	Yellowish-orange	Blue	–	
Vanillin	0.88	Orange	Blue	–	
Syringaldehyde	0.85	Pink	Blue	–	
(2) Sample	0.84	Pink	Blue	Purplish pink	
	0.67	Yellow-orange	Blue	–	+++
	0.63	Pink	Blue	–	+++
	0.32	Yellow	Blue	–	+++
p-hydroxybenzaldehyde	0.61	Yellowish	Blue	–	
Vanillin	0.67	Yellow-orange	Blue	–	
Syringaldehyde	0.63	Pink	Blue	–	
(3) Sample	0.89	Brown	Blue	–	++
	0.79	Orange	Blue	–	+++
	0.68	Brown	Blue	–	+++
	0.36	Yellowish-brown	–	–	+
	0.16	–	Blue	Blue	+

[a]Solvent (1)—Butanol:ethanol:water (5:1:4); Solvent (2)—Water saturated with benzene; Solvent (3)—Butanol:acetic acid:water (4:1:5).

Table 2—Thin layer chromatography data of oxidized stone cells

Compound	Solvent 1 Benzene:ethanol (150:22)			Solvent 2 Methanol:chloroform (3:7)			Solvent 3 Benzene:acetone (3:2)		
	Rf	Color[a]	Relative conc	Rf	Color	Relative conc	Rf	Color	Relative conc
Sample	0.96	Yellow	++				0.94	Yellow	+
	0.83	Orange-brown	+++	0.84	Orange-brown	++	0.86	Orange-brown	++
	0.80	Pinkish-brown	+++	0.82	Pinkish-brown	+++			
	0.76	Orange-brown	++	0.73	Brown	+			
	0.55	Brick red	++				0.59	Faint yellow	+
p-hydroxy-benzaldehyde	0.76	Orange-brown		0.87	Yellow-orange		0.88	Brown-orange	
Vanillin	0.82	Orange		0.84	Orange-brown		0.87	Orange-brown	
Syringaldehyde	0.80	Pinkish-orange		0.82	Pinkish-brown		0.81	Pinkish-orange	

[a]Color: Color formed when plates were sprayed with diazosulfanilic acid.

Enzymatic digestion of stone cells

Stone cells (1g) were suspended in 20 ml of citrate-phosphate buffer, pH 4.0 containing 5 mg each of cellulase and hemicellulase (Wallerstein, N.Y.) with continuous shaking at 40°C. The reaction was stopped after 4 hr by boiling the mixture for 5 min. The digest was filtered and the residue was washed with water and dried at 98°C for 3 hr and weighed. The dried cells were pulverized to pass through 120 micron sieve and again treated with enzymes as described above. The remaining residue was consecutively extracted with benzene, 95% ethanol, distilled water and sodium hydroxide (2N). The insoluble residue was filtered, washed with water, dried and weighed. The residue was then moistened with 1 ml N,N-dimethylaniline for 12 hr and then 20 ml 70% ice cold H_2SO_4 was added (Ryugo, 1969). The mixture was allowed to react at 4°C for 24 hr. At the end of this period, the reaction mixture was diluted with 200 ml of distilled water and boiled for 15 min. The mixture was then neutralized with saturated sodium hydroxide to a pH between 6 and 7 and filtered. The residue was again washed with distilled water, dried at 150°F for 5–6 hr and weighed.

Paper chromatography of sugars

Filtrates of the enzyme hydrolyzed stone cells were analyzed for sugars by paper chromatography. A 25–50 $\mu\ell$ sample was spotted on Whatman No. 1 paper, dried and the paper developed with butanol:acetic acid:water (4:1:5) for 18 hr. At the end of this period, the solvent front was marked and the paper dried in the air. The paper was then sprayed with aniline hydrogen phthalate (Partridge, 1949). Colored spots were marked with Rf values measured. Authentic samples of sugars were run simultaneously for comparison of Rf values with sample.

RESULTS & DISCUSSION

THE WIESNER reaction (Johansen, 1940) is not specific for lignin, itself, but for aldehyde groups of unpolymerized coniferyl aldehyde. Stone cells of pear turned red on treatment with this reagent and accordingly, appear to be ligninous in nature. This histochemical test was found useful in the present work to ascertain the purity of the isolated stone cells.

Oxidation of stone cells

The major oxidation products of stone cells were vanillin and syringaldehyde. Their identification was based on color reaction and mobility during paper chromatography (Table 1) and thin layer chromatography (Table 2). Besides these two major components, chromatographic studies showed the presence of four to five other separable zones which were not identified. These minor spots turned blue when sprayed with $K_3Fe(CN)_6$ + $FeCl_3$ reagent, indicating their phenolic nature. One zone which turned yellow on spraying with diazosulfanilic acid (Rf 0.91 and 0.32 in solvent 1 and solvent 2, respectively) was later found to be an oxidation product of nitrobenzene itself.

Three different solvent systems were used to carry out thin layer chromatography. Benzene-ethanol gave maximum separation of the sample with five spots and a very faint spot at the origin. Methanol-chloroform and benzene-acetone solvents gave only three spots each and a fairly dark spot at the origin.

It is known that nitrobenzene oxidation of soft woods yields mainly vanillin; whereas, hardwoods are mainly degraded to syringaldehyde and some vanillin. The lignin of grasses degrade to p-hydroxy-benzaldehyde, vanillin and syringaldehyde. The chromatographic data thus demonstrate the presence of lignin material analogous to that found in hardwoods.

Infrared spectroscopy of stone cells

The infrared spectra of stone cells (Fig. 1) showed extensive absorption at 1730 cm^{-1}. This is a region of absorption by carbonyl groups at the β-position. Bolkar and Terashima (1966) working on isolated wood lignin suggested that absorption in the region of 1700–1720 cm^{-1} is due to contribution from nonconjugated keto groups in the β positions of the phenylpropane side chain. The unconjugated β keto groups arise during iso-
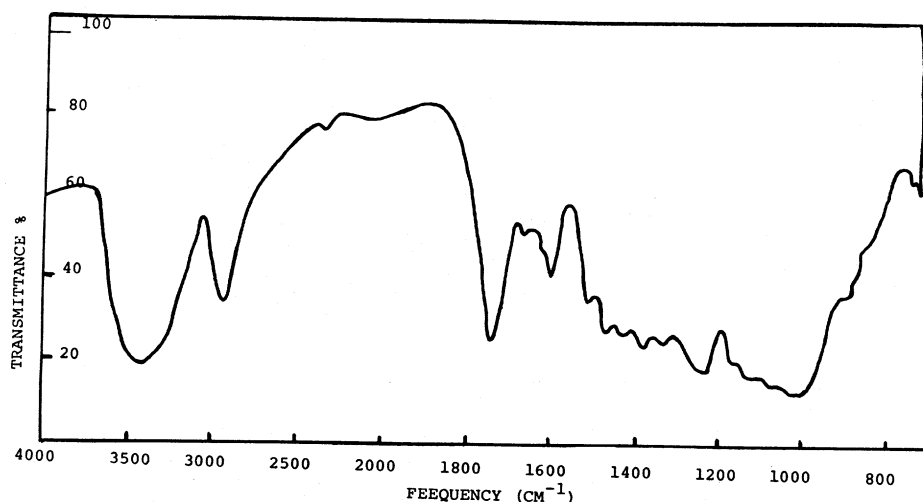

Fig. 1—Infrared absorption spectrum of stone cells.

Table 3—Carbohydrate material of pear stone cells

(A) Qualitative Tests of Enzyme Hydrolyzates

	Test	Observation	Inference
i.	Molisch reaction	Reddish violet ring produced at the interface	Carbohydrates present
ii.	Fehling solution test	Brick-red precipitate formed	Reducing sugars present
iii.	Seliwanoff reaction	Pinkish tinge	Ketose sugar absent
iv.	Phloroglucinol reaction	Chocolate-red color	Pentose, galactose or galactans present
v.	Bial Orcinol reaction	Green color formed in solution	Pentoses present

(B) Paper Chromatography of Enzyme Hydrolyzates

Solvent: Butanol:acetic acid:water (4:1:5)

Spot	Rf	Color with analine hydrogen phthalate	Inference
Sample	0.32	Pink	Xylose
	0.22	Brown	Glucose
	0.17	Pinkish-brown	Xylan
	0.13	Pinkish-brown	Xylan
Glucose	0.22	Brown	Glucose
Arabinose	0.28	Brown	Arabinose
Galactose	0.20	Brown	Galactose
Xylose	0.32	Pink	Xylose
	0.15	Pink	Xylan
	0.06	Pink	Xylan

lation of lignin from wood. Fukumuzi (1960) also observed greater absorption at wave number 1700 cm^{-1} for decayed wood lignin compared to that of Nord's lignin. He attributed this group because decayed lignin did not show purple red color with phloroglucinol-HCl reagent which is used to detect the conjugated coniferyl aldehyde group. The IR spectrum of stone cells was similar to Nord's decayed lignin (Fukumuzi, 1960) yet the stone cells reacted positively with phloroglucinol-HCl reagent.

The absorption bands (Fig. 1) at 1510 cm^{-1} and 1590 cm^{-1} indicate the phenyl ring skeletal vibrations with possible para substitution. The band at 1420 cm^{-1} may be due to the presence of aliphatic structure. The band at 2946 cm^{-1} is due to CH groups.

Cellulase, hemicellulase digestion of stone cells

Intact stone cells were first digested with cellulytic enzymes to remove adhering cell wall fragments not removed by washing. The observed loss in weight was 27.4%. The undigested material was considered to represent pure stone cells.

Treated stone cells were then pulverized and again treated with carbohydrases. An additional 15.3% of their weight was solubilized on this second treatment. The digested powder was extracted with benzene, ethanol and alkali with a further weight loss of 41.1%. Finally, treatment of the residue with 70% H_2SO_4 to remove additional cellulosic material resulted in 25.9% loss in weight on the basis of the original material. The residue remaining after these treatments was 17.7% of the original stone cell. This residue also gave a positive reaction for lignin when treated with phloroglucinol-HCl. The 82% weight loss resulting from these treatments presumably represented the cellulosic constituents, analogous to those found in wood lignin, as well as lesser quantities of lipid and protein substances.

Analysis of carbohydrates during enzyme digestion

Qualitative test showed the presence of reducing sugars, hexoses and pentoses in the enzyme hydrolyzate (Table 3A). The principle sugars in this fraction were identified as glucose and xylose and

xylans of varied degrees of polymerization (Table 3B). These sugars are representative monomers of cellulose and hemicellulose material associated with woody plant tissues (Ling and Nanji, 1923).

CONCLUSIONS

RESULTS reported here demonstrate that stone cells are lignocellulosic, containing approximately 18% lignin and 82% of a material which was principally carbohydrate. The principal monomer units of lignin were vanillin and syringaldehyde and the carbohydrate hydrolyzate contained glucose and xylose residues.

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